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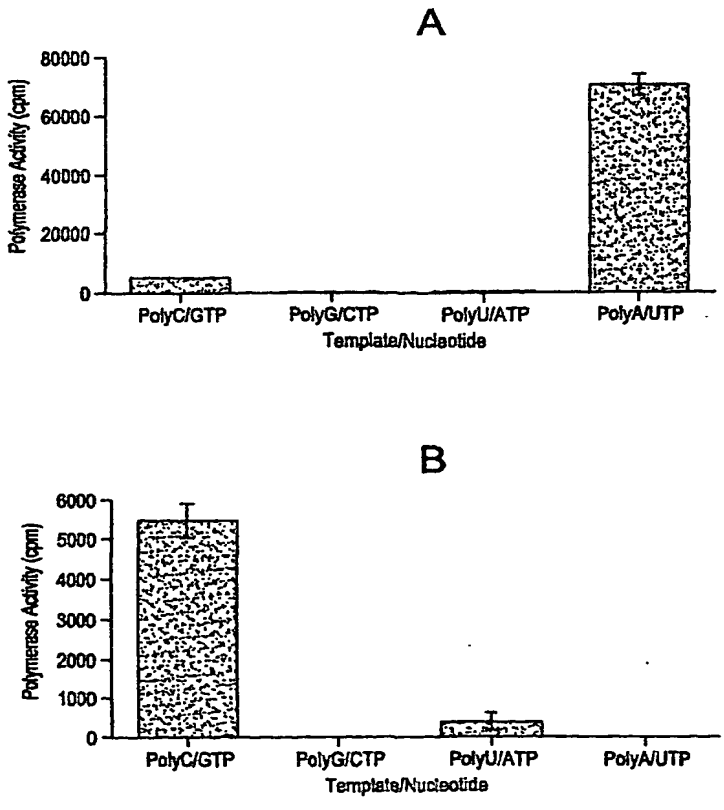
(54) Title: PRIMER–INDEPENDENT RNA SYNTHESIS CATALYZED BY HCV POLYMERASE

(57) Abstract

Provided are methods for identifying inhibitors of primer–independent hepatitis C virus RNA–dependent RNA polymerase *de novo* RNA synthesis activity, methods for inhibiting the polymerase using such inhibitors, and methods for inhibiting hepatitis C virus replication. These methods employ homopolyC templates; homopolyU templates; synthetic heteropolymeric RNA templates comprising a cluster of cytidylate nucleotides, a cluster of uridylate nucleotides, or a mixed cluster of cytidylate and uridylate nucleotides; and synthetic or naturally occurring RNA templates comprising an RNA synthesis initiation pyrimidine nucleotide. Reactions can be carried out in buffers containing Mn^{++} alone, or in combination with Mg^{++} ions.



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PRIMER-INDEPENDENT RNA SYNTHESIS CATALYZED BY HCV POLYMERASE

This application claims the benefit of priority of U.S. Provisional Applications Serial Nos. 60/111,773, filed December 10, 1998, and 60/111,825, filed December 11, 1998, respectively, the contents of each of which are herein incorporated by reference in their entirety.

Background of the Invention

Field of the Invention

The present invention relates to the field of human medicine, particularly the discovery of new anti-viral agents.

Description of Related Art

Hepatitis C virus (HCV) is a major causative agent of non-A, non-B hepatitis. Its persistent infection causes chronic hepatitis in humans with a high frequency of liver cirrhosis, and is strongly associated with the development of hepatocellular carcinoma. As a member of the flavivirus family, HCV is an enveloped virus with a single-strand, positive sense RNA genome of approximately 9.6 kb.

Little is known about the HCV replication mechanism due to the lack of an efficient cell culture system, although an animal model has been established recently (Kolykhalov, A. A. et al., *Science* 277, 570-574 (1997); Yanagi, M. et al. *Proc. Natl. Acad. Sci. USA* 94, 8738-8743 (1997)). To date, most studies have concentrated on investigation of the functions of HCV proteins and enzymes. The HCV genome encodes at least 10 structural and non-structural viral proteins, which are generated from maturational cleavage of

the polyprotein precursor (Reed, K.E. et al. in *Hepatitis C virus*, Ed. Reesink HW (Karger, Basel), pp.1-37 (1998); Clarke, B. J. Gen. *Virology* 78, 2397-2410 (1997); Houghton, M. in *Virology*. Eds. Fields, B. N., Knipe, D. M. Howley P. M. (Raven Publishers. Philadelphia), pp1035-1058 (1996)). One of the non-structural proteins, designated NS5B, is an RNA-dependent RNA polymerase. As one of the key components implicated in viral replication (Reed, K.E. et al. in *Hepatitis C virus*, Ed. Reesink HW (Karger, Basel), pp.1-37 (1998); Clarke, B. J. Gen. *Virology* 78, 2397-2410 (1997); Houghton, M. in *Virology*. Eds. Fields, B. N., Knipe, D. M. Howley P. M. (Raven Publishers. Philadelphia), pp1035-1058 (1996); Miller, R. H. et al. *Proc. Natl. Acad. Sci. USA* 87, 2057-2061 (1990)), the HCV NS5B enzyme has received a great deal of attention. The recombinant HCV NS5B protein has been purified and characterized only recently (Behrens, S. E. et al. *EMBO J.* 15, 12-22 (1996); Lohmann, V. et al. *J Virol.* 71, 8416-8428 (1997); Yamashita, T. et al. *J. Biol. Chem.* 273, 15479-15486 (1998); Lohmann, V. et al. *Virology* 249, 108-118 (1998); Lohmann, V. et al. *J. Biol. Chem.* 274, 10807-10815 (1999); Johnson, R. B. et al. Submitted *to J. Biol. Chem.* (1999)). This enzyme has been found to catalyze RNA elongation in which nucleotidyl residues could be transferred by the polymerase to the 3'-hydroxyl of an RNA primer that is hybridized to a template. It is most likely that the viral replicase complex catalyzes *de novo* initiation of RNA synthesis in infected cells.

The HCV genome consists of a 5'-untranslated region (UTR) of ~340 nucleotides, a long single open reading frame that typically encodes a polyprotein of about 3000 amino acids, and a 3'-UTR with a short sequence of nucleotides (~28 to 42) followed by a poly(U)/polypyrimidine tract of variable length, and an additional 98 nucleotides that show conservation in divergent HCV genotypes. See, for example,

Yamada N. et al., *J. Gen. Virol.* vol. 75, pp 3279-3284 (1994); Tanaka et al., *Biochem Biophys Res Commun.*, vol. 215, pp 744-749 (1995); Tanaka T et al., *J Virol.* vol. 70, pp 3307-3312 (1996); Yamada N. et al., *Virology*, vol. 223 pp 255-261 (1996); and Kolykhalov et al., *J. Virol.*, vol. 70, pp 3363-3371 (1996).

The single large HCV polyprotein precursor translated from the HCV genome is processed by host signal peptidase and two viral proteinases to generate mature viral proteins and functional enzymes. These include three structural proteins, the p22 core protein and two glycoproteins (E1 and E2), and at least seven nonstructural proteins, in the order of NS2A-NS2B-NS3-NS4A-NS4B-NS5A-NS5B. See, for example, Bradley DW, *Transfus Med Rev.* vol. 6, pp 93-102 (1992); Shimotohno K. et al., *Princess Takamatsu Symp.*, vol. 25, pp 121-128 (1995); Brechot C., *Dig Dis Sci.*, 41:6S-21S (1996); Lohmann V. et al., *J. Hepatol.*, vol. 24, pp 11-19 (1996); and Neddermann P. et al., *Biol Chem.*, vol. 378, pp 469-476 (1997).

Of the nonstructural proteins, NS5B is predicted, based on its amino acid sequence, to be an RNA-dependent RNA polymerase that is implicated in viral replication. Miller R.H. et al., *Proc Natl Acad Sci USA*, vol. 87, pp 2057-2061 (1990). Specifically, it is believed that the NS5B polymerase is an important component of the replicase complex for viral genome replication. Recombinant polymerase has been shown to synthesize RNA in a primer-dependent elongation manner. However, initiation of RNA synthesis in HCV infected cells is likely to occur via a *de novo* initiation mechanism. Until recently, *de novo* initiation has not been reported with this or other viral RNA-dependent RNA polymerases.

Kao, C.C. et al. *Virology*, vol. 253, pp 1-7 (1999) reported initiation of RNA synthesis by a *de novo* mechanism

from templates using recombinant RNA-dependent RNA polymerase NS5B of bovine viral diarrhea virus (BVDV), a positive strand RNA virus in the Flaviviridae family. The template used in this study was a short, chemically synthesized RNA, denoted "(-)21g," that corresponds to the 21 nucleotides (nt) at the 3' end of the negative strand BVDV genome, presumably containing the signals for the initiation of genomic positive strand synthesis. To discern *de novo* initiation from primer extension, these workers added a guanylate modified to have a dideoxyribose to the 3' end of the BVDV 21-nt negative strand to form the (-)21g template. The lack of 2' and 3' hydroxyl groups rendered the template incapable of priming RNA synthesis. According to the authors, changing the penultimate (the +1) cytidylate to either a guanylate or a uridylate was detrimental, drastically reducing RNA synthesis, and suggesting that the +1 cytidylate (the 3'-most nucleotide in the unmodified, natural template) is the initiation nucleotide. The authors concluded that *de novo* initiation of positive strand BVDV RNA synthesis by recombinant BVDV RNA-dependent RNA polymerase NS5B can take place from an internal initiation nucleotide, i.e., a penultimate cytidylate residue at the 3' end of the negative strand template. The authors also suggested that initiation of RNA synthesis by BVDV NS5B may prefer a cytidylate as the 3' nucleotide, although a cytidylate at the penultimate position is also acceptable. The authors also concluded that recognition of the initiation site requires a cytidylate present at or near the 3' end of the template. A template competition assay designed to determine whether nucleotide changes from (-21)g affect the ability to interact with BVDV NS5B polymerase demonstrated that change of the initiation cytidylate to a uridylate (+1C/U) resulted in a better competitor in comparison to +1C/G. The results suggested that NS5B was able to bind to the RNA containing a uridylate at the +1 position, but was unable to direct RNA synthesis as efficiently as when a +1 cytidylate is present.

Furthermore, extensions to the 3' end of the template had a detrimental effect on the ability of the template to interact with the polymerase. Finally, the authors noted that template length may affect the mode of initiation of RNA synthesis observed (3' extension or primer-dependent elongation vs. *de novo* initiation), and that the initiation of RNA synthesis from the sequences directing negative strand RNA synthesis may be different from those directing the initiation of positive strand RNA synthesis, i.e., there are different requirements in the templates for negative and positive strand synthesis.

Oh, J-W et al., *Jour. Virol.*, vol. 73, pp 7694-7702 (1999) discloses a recombinant HCV 1b NS5B capable of initiating RNA synthesis *de novo* and yielding a full-length RNA product of genomic size (9.5 kb) in a template-dependent and primer-independent manner. Use of the conserved 3'-end 98 nucleotides of plus strand HCV RNA as a template is described. Truncation of 21 nucleotides from the 5' end, or 45 nt from the 3' end, of the 98 nucleotide RNA almost completely abolished template activity. Inclusion of the 3'-end variable sequence region and the U-rich tract upstream of the X region in the template significantly enhanced RNA synthesis. The 3' end of minus strand RNA of the HCV genome also served as a template, requiring a minimum of 239 nucleotides from the 3' end. In the presence of complementary oligonucleotide primers, ^{32}P-ribonucleotides were incorporated into TCA-precipitable when poly(A) or poly(C) RNA was used as the template. In contrast, poly(G) and poly(U) templates were very weak templates in the presence of a primer. In all four cases, very little RNA synthesis was detected in the absence of a primer.

An understanding of the mechanism of action of the NS5B polymerase would facilitate the identification of compounds

capable of interfering with HCV replication, thereby providing a means for treating and/or preventing HCV infection.

Summary of the Invention

The present inventors have surprisingly discovered that purified recombinant HCV polymerase can initiate *de novo* RNA synthesis in a primer-independent manner using RNA as a template by recognizing cytidylate or uridylate pyrimidine nucleotides present in the template. This discovery facilitates study of the mechanism of HCV replication, in particular, the polymerase initiation requirement. For example, this discovery can be used to develop an assay to identify inhibitors of RNA initiation catalyzed by the polymerase, discussed in detail below.

Accordingly, in a first aspect, the present invention provides a method of identifying a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase, comprising:

a) contacting an RNA template comprising an RNA synthesis initiation pyrimidine nucleotide with a flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the absence of the compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

b) contacting an RNA template as in a) with the flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the presence of the compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

c) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase.

In this and the following methods, the RNA template can be a synthetic RNA sequence, for example a sequence comprising a sequence selected from a homopolyC template; a homopolyU template; a synthetic heteropolymeric RNA template comprising a cluster of cytidylate nucleotides, a cluster of uridylate nucleotides, or a mixed cluster of cytidylate and uridylate nucleotides; or a synthetic or naturally occurring RNA template comprising an RNA synthesis initiation pyrimidine nucleotide, e.g., a cytidylate or uridylate moiety. In these cases, the RNA synthesis initiation pyrimidine nucleotide can be a cytidylate nucleotide or a uridylate nucleotide. In this and the following methods, the RNA template can also comprise hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, such as the 3′ untranslated region of positive or negative RNA strand hepatitis C virus genomic RNA. It should be noted that in this and the following methods, the fragment can comprise the 3′ untranslated region of positive or negative strand hepatitis C virus genomic RNA, or it can consist essentially of, or consist of, this fragment. It should also be noted that in this and the following methods, reaction buffers can contain, in addition to other necessary cofactors and reagents, Mn^{++} alone, or Mn^{++} in combination with Mg^{++} ions. A preferred concentration range for Mn^{++} salts is in the range of from about 0.5 mM to about 20 mM, more preferably from about 5 mM to about 10 mM.

When using hepatitis C virus genomic RNA or a fragment thereof, the RNA synthesis initiation pyrimidine nucleotide can be a uridylate nucleotide.

In this and the following methods, the flavivirus RNA-dependent RNA polymerase can be a hepatitis virus RNA-dependent RNA polymerase, particularly that of a hepatitis C virus, wherein the hepatitis C virus can be selected from hepatitis C virus genotype 1a, hepatitis C virus genotype 1b, hepatitis C virus genotype 2a, hepatitis C virus genotype 2b, progeny of any of the foregoing, and a mutant of any of the foregoing. Other flaviviruses useful in the present invention are disclosed by Simmonds, P. in *Hepatitis C Virus*, Second Edition, H.W. Reesink, ed., Karger, NY (1998). In a preferred embodiment, the hepatitis C virus is a hepatitis C virus genotype 1b virus, or any progeny or mutant thereof, and the hepatitis C virus RNA-dependent RNA polymerase is hepatitis C virus NS5B polymerase, which can be produced by recombinant means.

In another aspect, the present invention provides a method of identifying a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase, comprising:

a) contacting an RNA template comprising hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, with hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the absence of the compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

b) contacting the RNA template as in a) with the hepatitis C virus NS5B RNA-dependent RNA

polymerase in the absence of a primer and in the presence of the compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

c) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase.

In any of the foregoing or following methods, the contacting of steps a) and b) can carried out *in vitro* or *in vivo*.

In another aspect, the present invention provides a method of inhibiting primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase, comprising contacting the flavivirus RNA-dependent RNA polymerase with a polymerase-inhibiting effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by the polymerase,

wherein the compound is identified by a method comprising:

a) contacting an RNA template comprising an RNA synthesis initiation pyrimidine nucleotide with a flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the absence the compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

b) contacting an RNA template as in a) with the flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the presence of the compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

c) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase.

In another aspect, the present invention provides a method of inhibiting primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase, comprising contacting the hepatitis C virus RNA-dependent RNA polymerase with a polymerase-inhibiting effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by the polymerase,

wherein the compound is identified by a method comprising:

a) contacting an RNA template comprising hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, with hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the absence of the compound under conditions

permitting RNA synthesis, and determining the amount of RNA product thus formed;

b) contacting the RNA template as in a) with the hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the presence of the compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

c) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase.

In yet another aspect, the present invention provides a method of inhibiting replication of a flavivirus, comprising contacting the flavivirus with an antiviral effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerase,

wherein the compound is identified by a method comprising:

a) contacting an RNA template comprising an RNA synthesis initiation pyrimidine nucleotide with a flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the absence the compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

b) contacting an RNA template as in a) with the flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the presence of the compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

c) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerase.

In a still further aspect, the present invention provides a method of inhibiting the replication of hepatitis C virus, comprising contacting the hepatitis C virus with an antiviral effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase,

wherein the compound is identified by a method comprising:

a) contacting an RNA template comprising hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, with hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the absence of the compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

b) contacting the RNA template as in a) with the hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the presence of the compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

c) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase.

It should be understood that the present invention encompasses all appropriate combinations of the particular and preferred groupings disclosed herein.

Further scope of the applicability of the present invention will become apparent from the detailed description provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the present invention, are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

The above and other aspects, features, and advantages of the present invention will be better understood from the following detailed description taken in conjunction with the accompanying drawings, all of which are given by way of

illustration only and are not limitative of the present invention, in which:

Figures 1A and 1B show *in vitro* RNA synthesis catalyzed by purified HCV NS5B using homopolymer RNAs as templates and corresponding α-^{32}P labeled nucleotide substrates as indicated. Reactions were performed for 4 hrs using 10 μM nucleotide, 10 μg/ml RNA template, and 2 μg/ml purified HCV NS5B enzyme in the presence (panel A) or absence (panel B) of the corresponding primer. 1 μg/ml complementary primer was used in the reactions containing primer.

Figures 2A and 2B show the enzyme-concentration-dependent and time-dependent course of *de novo* RNA synthesis catalyzed by purified recombinant HCV NS5B. In each RNA synthesis reaction, polyC was used as a template along with 100 μM of GTP (mixed with α-^{32}P-GTP) as the substrate. Reactions were run in triplicate. Panel A: dose dependence of primer-independent RNA synthesis catalyzed by HCV NS5B. The reactions were carried out at room temperature for 1 hr with the specified enzyme concentration. Panel B: time progress of primer-independent RNA synthesis catalyzed by HCV NS5B. The reactions were incubated with 50 ng NS5B at room temperature. At each indicated time point, an aliquot was taekn out and the reaction was terminated as indicated. A control with no NS5B was performed under identical conditions.

Figure 3A shows direct evidence of *de novo* RNA synthesis initiation catalyzed by HCV NS5B. The RNA synthesis reactions were carried out for 4 hr using polyC as template and GTP as the substrate at a concentration of 20 μM mixed with γ-^{32}P-GTP (250 mCi/ml). The data show that the RNA products could be labeled by ^{32}P when γ-^{32}P-GTP was used

as substrate in the reaction, indicating the presence of triphosphate GTP in the products, and suggesting that the purified NS5B enzyme synthesizes RNA by a *de novo* mechanism. No RNA product was identified when polyA was used as template under the identical conditions.

Figure 3B shows the time course of RNA synthesis by NS5B in the absence of primer. Reactions (-•-) were performed for the time specified using 10 μm GTP/[α-^{32}p]-GTP, 0.5 μg/ml polyC, and 1 μg/ml purified HCV NS5B in the absence of primer. Reactions containing polyA template with UTP substrate (-■-) in the absence of primer were run as controls.

Figure 4 shows a kinetic analysis of primer-dependent and primer-independent RNA synthesis catalyzed by purified NS5B polymerase as a function of substrate concentration. The RNA synthesis reactions were carried out with different concentrations of substrate GTP (mixed with α-^{32}P-GTP). The reactions were incubated at room temperature for 4 hr.

Figure 5 shows HCV NS5B catalyzed *de novo* RNA synthesis from HCV RNA template. Panel A: Schematic representing the HCV RNA fragment used in Example 3. Panel B: RNA synthesis catalyzed by HCV NS5B from an HCV RNA template. Reactions were performed using 1 μg/ml HCV RNA template under the conditions described. Labeled nucleotide was used at 0.6 μM (specific activity: ~800 Ci/mmol) and the remaining three nucleotides were at 10 μM each. Lane 1, labeled RNA template only; lanes 2 and 3, unlabeled template with [α-^{32}P]-ATP and [α-^{32}P]-UTP, respectively. Panel C: HCV polymerase reaction using unlabeled nucleotides. Reactions were performed for 2 hrs using unlabeled NTP substrates (10 μM each) but with the ^{32}P-labeled HCV RNA template (1 μg/ml). Radiolabeled RNA products were analyzed by

sequencing gels followed by autoradiography. The positions for molecular markers are indicated.

Figure 6 shows HCV NS5B initiated *de novo* RNA synthesis by recognizing a uridylate present in the HCV RNA template. The purified HCV NS5B polymerase catalyzed incorporation of ATP as the first nucleotide into the nascent RNA products. Reactions were carried out for 2 hrs and analyzed as described in the legend to Figure 5 using either [γ-^{32}P]-ATP or [γ-^{32}P]-GTP (1 μM with specific activity of 1,500 Ci/mmol) as the labeled substrate.

Figure 7 shows the effect of Mg^{2+} and Mn^{2+} on the primer-dependent/independent RNA synthesis catalyzed by purified HCV NS5B enzyme. Reactions were performed for 1 hr at room temperature under the conditions described in Example 4.

Detailed Description of the Invention

The following detailed description of the invention is provided to aid those skilled in the in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited herein are herein incorporated by reference in their entirety.

Conventional methods of gene isolation, molecular cloning, vector construction, etc., are well known in the

art and summarized, for example, in Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. One skilled in the art can readily reproduce the plasmid vectors described herein without undue experimentation employing these methods. The various nucleic acid sequences, fragments, etc., necessary for this and other purposes can be readily obtained as components of commercially available plasmids, or are otherwise well known in the art and publicly available, or readily reproducible based upon published information.

As used herein, the term "primer" means a nucleic acid fragment of any size that functions as an initiating substrate for enzymatic or synthetic elongation of, for example, a nucleic acid molecule, such as an RNA or DNA molecule. The nucleotides in the primer must be complementary to nucleotides in the template.

The term "initiation cytidylate nucleotide" means a cytidine nucleotide or a nucleotide sequence comprising at least one cytidine moiety. The initiation cytidylate nucleotide functions as a recognition site for primer-independent *de novo* RNA synthesis on an RNA template catalyzed by flavivirus RNA-dependent RNA polymerase.

The term "initiation uridylate nucleotide" means a uridine nucleotide or a nucleotide sequence comprising at least one uridine moiety. The initiation uridylate nucleotide functions as a recognition site for primer-independent *de novo* RNA synthesis on an RNA template catalyzed by flavivirus RNA-dependent RNA polymerase.

The terms "complementary" or "complementarity" as used herein refer to the capacity of purine and pyrimidine nucleotides to associate through hydrogen bonding to form double stranded nucleic acid molecules. The following base pairs are related by complementarity: guanine and cytosine; adenine and thymine; and adenine and uracil. Complementarity can be full, i.e., involving all base pairs comprising two single-stranded nucleic acid molecules, or partial.

"Substantially purified" means a specific isolated nucleic acid or protein, or fragment thereof, in which substantially all contaminants, i.e., substances that differ from the specific nucleic acid or protein molecule, have been removed therefrom. For example, a protein may, but not necessarily, be "substantially purified" by the column chromatographic methodology, described herein. "Substantially purified" can refer to molecules that are about 75%, about 80%, about 85%, about 90%, about 95%, or about 99% or more pure.

The term "plasmid" refers to an extrachromosomal genetic element. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accordance with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Recombinant DNA cloning vector" as used herein refers to any autonomously replicating agent, including, but not limited to, plasmids and phages, comprising a DNA molecule to which one or more additional DNA segments can or have been added.

The term "vector" as used herein refers to a nucleic acid compound used for introducing exogenous DNA into host cells. A vector comprises a nucleotide sequence which can encode one or more protein molecules. Plasmids, cosmids, viruses, and bacteriophages, in the natural state or which have undergone recombinant engineering, are examples of commonly used vectors.

As noted above, the present invention discloses a new mechanism for initiating RNA synthesis by HCV polymerase. As one of the key components implicated in viral replication, polymerase represents an important target for inhibiting HCV, thus providing methods for identifying compounds that can be used to treat and/or prevent HCV infections.

Recombinant HCV polymerase has been purified and characterized. See, Behrens S.E. et al., *EMBO J.*, vol. 15, pp 12-22 (1996), Lohmann V. et al., *J. Virol.*, vol. 71, pp 8416-8428 (1997); and Yuan Z.H. et al., *Biochem Biophys Res Commun.*, vol. 232, pp 231-235 (1997). The polymerase catalyzes an RNA elongation reaction in which nucleotidyl residues are transferred by the polymerase to the 3'-hydroxyl of an RNA primer hybridized to the template. Recombinant polymerase has been reported to catalyze the RNA synthesis reaction in a primer-dependent manner (Behrens et al., 1996; Lohmann et al., 1997; and Yuan et al., 1997).

The present invention discloses that purified polymerase initiates *de novo* RNA synthesis in a primer-independent manner in the presence of a homopolyC template, a homopolyU template, or other template containing an initiation cytidylate (C) or uridylate (U) nucleotide, i.e., an initiation pyrimidine base. The data disclosed herein also provide evidence that purified HCV polymerase catalyzes *de novo* RNA synthesis in a primer-independent manner using

HCV RNA as well as these homopolymers as templates. With HCV RNA as the template, the resulting nascent RNA products, smaller than the template used, contained ATP as the first nucleotide. These results indicate that the newly synthesized RNAs did not result from template self-priming, and suggest that a replication initiation site in the HCV RNA genome is a uridylate.

These observations have been used to develop assays using homopolyC, homopolyU, mixed polyC/polyU, HCV RNA and fragments thereof, and other nucleic acids (both RNA and DNA) as templates for RNA synthesis. These assays can be used to identify compounds that inhibit primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerases such as the HCV polymerase. Inhibitor compounds so identified can be used to inhibit primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerases such as the HCV polymerase *in vitro* and *in vivo*, and to inhibit the replication of flaviviruses such as HCV *in vitro* and *in vivo*.

Identification of Compounds That Inhibit Primer-Independent *de novo* RNA Synthesis Catalyzed by RNA-Dependent RNA Polymerase

The existence of *de novo* RNA synthesis initiation catalyzed by HCV polymerase can be used to identify the RNA replication initiation site in the viral genome, thus increasing knowledge concerning the mechanism of HCV genome replication *in vivo*. In addition, understanding the RNA-dependent RNA polymerase catalytic mechanism disclosed herein can be used to design assays for identifying compounds that inhibit HCV polymerase, particularly the primer-independent RNA synthesis initiation reaction catalyzed by the polymerase.

For example, the present invention contemplates an assay employing polyC as a template to search for HCV polymerase inhibitors. In contrast to other measurements of polymerase activity based on its elongation reaction, such an assay would permit identification of inhibitors that specifically block the first step of RNA synthesis initiation catalyzed by HCV NS5B and related RNA-dependent RNA polymerases.

Thus, the present invention provides a method of identifying an HCV polymerase inhibitor, which comprises:

a) exposing HCV polymerase to a test compound in the presence of a homopolyC template or a template containing an initiation cytidylate nucleotide; and

b) measuring the RNA product by any suitable means.

For example, in one embodiment, the polymerase is purified, and used in a screen to identify a test compound that inhibits the RNA synthesis initiation reaction of the polymerase. A variety of suitable screens for assaying test compounds that inhibit this initiation reaction are contemplated for this purpose, for example, using various labeling techniques, such as radiolabeling or fluorescent tagging, or by labeling with biotin/avidin. Thereafter, inhibition can be determined by any suitable means known in the art.

The screening methods of the present invention can be adapted to automated procedures such as a PANDEX® (Baxter-Dade Diagnostics) system, allowing for efficient high-volume screening of HCV inhibitor compounds.

Non-limiting examples of materials and methods useful in practicing the present invention are as follows.

Materials—Template homopolymer RNAs were obtained from Pharmacia, and heteropolymer RNAs were purchased from Sigma.

Oligonucleotide RNA primers were custom designed and synthesized by CyberSyn (Lenni, PA). Radiolabeled nucleotides were purchased from Amersham and NEN. *In vitro* transcription kits containing pSPT-18 and pSPT-19 plasmids were purchased from Roche (Indianapolis, IN). Nitrocellulose filter membrane was from Schleicher & Schuell. 20xSSC buffer, 10x TBE, 10X PBS, and other molecular biology reagents including molecular markers, ribonucleotides, T4 polynucleotide kinase, ligase, and restriction endonucleases were obtained from Gibco and New England BioLabs. Protein purification resins and pre-packed columns were from Pharmacia. Recombinant HCV genotype 1b NS5B polymerase was expressed in *E. coli* using a cDNA clone, and purified by sequential chromatographic columns, as described below.

Expression and purification of recombinant HCV NS5B polymerase—*E. coli* BL21-DE3 was transformed with expression vector pRSET-A (Invitrogen) carrying HCV genotype 1b NS5B cDNA by heat shock (40 sec at 42°C). Any mutein thereof, or other RNA-dependent RNA polymerase possessing HCV primer-independent RNA-dependent RNA polymerase activity, can also be used to produce active recombinant HCV polymerase useful in the present invention. Other suitable HCV genotype or subtype cDNAs can be used to produce suitable HCV polymerase, for example, HCV genotype 1a, genotype 2a, or any mutant thereof. Other suitable HCV subtypes and genotypes are disclosed in Simmonds P., *Clinical Therapeutics*, vol. 18, pp 9-36 (1996) and Brechot C., *Digestive Diseases and Sciences*, vol. 41, pp 6S-21S (1996).

The transformed bacterial cells were cultured at 25°C to an OD_{600} of 0.6 and then induced by 0.5mM IPTG for 4hr to produce recombinant polymerase. The harvested cells were washed in 1X PBS buffer and resuspended in lysis buffer (20mM Tris pH 7.5, 1mM EDTA, 10mM DTT, 10% glycerol) with 2% Triton X-100, 0.5M NaCl, 10mM $MgCl_2$, with protease inhibitors and DNase I. After sonication on ice TOTAL 5 MIN

10X30 SEC30 SEC ON 60 SEC, the crude extract was centrifuged at 100,000 x g for 30 minutes and then the supernatant was collected and diluted with lysis buffer to 0.3M NaCl.

The supernatant containing the soluble NS5B protein was incubated with DEAE resin at 4°C for 2hr, and the flow-through was loaded on a HiTrap Heparin sepharose column (Pharmacia). The column was washed with buffer A (25mM HEPES pH 7.5, 1mM EDTA, 10mM DTT, 20% glycerol, 0.5% Triton X-100) containing 0.3M NaCl, and the bound proteins were eluted with a gradient of 0.3M to 1M NaCl in buffer A at 1 ml/min. RNA-dependent RNA polymerase activity in the fractions was identified by the filter assay described below in the presence of rifampicin. Active fractions were collected, diluted with Buffer A at a 1:5 ratio to reduce its ionic strength, and loaded to a poly-U sepharose column. After washing with 0.15M NaCl in buffer A, proteins were eluted using a gradient of 0.15-0.7M NaCl. Fractions were analyzed by the polymerase filter assay. The active fractions were pooled and diluted with buffer B (25mM Hepes, pH8.0, 1mM EDTA, 10mM DTT, 20% glycerol, 0.5% Triton X-100) at a ratio of 1:10. The diluted sample was loaded onto a HiTrap Q column (Pharmacia) and the flow-through containing NS5B protein was collected. Purified recombinant polymerase was analyzed by SDS-PAGE and Western blot using polyclonal anti-NS5B antibodies developed in. A single 65kD protein band was present in the sample; its identity as HCV NS5B was confirmed by N-terminal amino acid sequencing.

RNA synthesis assays for HCV polymerase—The incorporation of ^{32}P-labeled nucleotides was measured substantially as described in Oberste et al., 1988, *Nucleic Acids Res.*, vol. 16, pp 10339-10352 (1988); Behrens et al., 1996; Lohmann et al., 1997). A typical NS5B polymerase assay was performed using homopolymer RNA (polyA) as the template and a 12-mer oligo-U_{12} as the primer in a reaction buffer (20µl) containing 25mM Tris-HCl, pH 7.5, 5mM $MgCl_2$,

25mM KCl, 1mM DTT, 10μM UTP, 50 mCi/ml [α-^{32}P] UTP, 10 μg/ml polyA, 1 μg/ml oligo-U_{12}, and 50ng purified recombinant NS5B. After one hour incubation at room temperature, the reaction was terminated by the addition of 0.2ml of 50mM EDTA and then loaded onto a nitrocellulose membrane using a 96-well minifold. The membrane was washed in 2X SSC buffer to remove the unused radiolabeled UTP substrate. Radioactivity bound to the filter membrane was measured and quantitated by a scintillation counter.

The primer-independent RNA synthesis reaction for NS5B polymerase was carried out under the foregoing conditions unless otherwise specified with various RNA template, but in the absence of any primers. Various templates were used, such as polyAC, polyCU, and homopolymer RNA polyC, and the corresponding nucleotides with appropriate radiolabeling (for example, α-^{32}P-GTP or γ-^{32}P-GTP) was used as substrate as indicated. The polymerase reactions were performed at room temperature. At each indicated time point, the reaction was terminated as described above. Incorporation of the radiolabeled nucleotide substrate was quantitated by scintillation counting.

Example 1

De novo Initiation of RNA Synthesis by Purified Recombinant HCV NS5B In the Absence of a Primer

Using the reaction conditions described above, *de novo* initiation of RNA synthesis catalyzed by HCV polymerase was observed when polyC and polyU were used as the template in the absence of a primer (Figure 1B). In contrast, in the absence of an appropriate oligonucleotide primer, no radiolabeled nucleotide substrate incorporation was found in assays employing polyA or polyG as templates. These results also exclude the possibility of terminal transferase activity associated with the NS5B polymerase.

To confirm further that a cytidylate nucleotide residue can be recognized by the HCV polymerase for initiation, RNA templates such as polyAC and polyCU, which contained cytidylate residues, were tested. In all cases, incorporation of radiolabeled GTP into the RNA reaction products was observed, although the efficiency was lower than when polyC was used as the template. These results demonstrate that the polymerase can initiate RNA synthesis when cytidylate residues are present in the template.

Taken together, these results suggest that HCV RNA-dependent RNA polymerase can utilize cytidylate and uridylate residues for primer-independent initiation of *de novo* RNA synthesis.

The primer-independent RNA synthesis reaction catalyzed by the polymerase can be carried out at a temperature of about 4°C to about 42°C. The reaction is preferably carried out a temperature of about 22°C to about 32°C. The preferred temperature is about 30°C.

As shown in Figures 2A and 2B, respectively, the primer-independent RNA synthesis reaction catalyzed by the polymerase incorporated radiolabeled nucleotide substrate into the RNA products in an enzyme-concentration-dependent and time-dependent manner. To exclude the possibility that the RNA synthesis catalyzed by NS5B was directed by RNA or DNA contaminating the NS5B polymerase preparation, the α-^{32}P-labeled GTP substrate was changed to γ-^{32}P-labeled GTP. If the observed RNA synthesis was directed by any contaminating RNA or DNA primer, the resulting RNA products would not be labeled by γ-^{32}P-GTP since the RNA synthesis elongation reaction could only incorporate GMP into the growing RNA products. However, if the RNA products were synthesized by a *de novo* mechanism, the RNA products would be ^{32}P-labeled since the first residue of the resulting RNA

should be a triphosphate GTP residue. As shown in Figure 3, the RNA products could be labeled by ^{32}P when γ-^{32}P-GTP was used as substrate in the reaction, indicating the presence of triphosphate GTP in the products, and suggesting that the purified NS5B enzyme synthesizes RNA by a *de novo* mechanism. No RNA product was identified when polyA was used as template under the identical conditions.

Example 2

Kinetic Analysis of Primer-Dependent and Primer-Independent RNA Synthesis Catalyzed by Purified HCV Polymerase

It has been reported that the BMV RNA-dependent RNA polymerase required high GTP substrate concentration to initiate RNA synthesis. See, Kao C.C. et al., *J. Virol.*, vol. 70 pp.6826-6830 (1996). To test if this is true for the HCV polymerase, the rates of polymerase-catalyzed RNA synthesis by elongation of the 3'-terminus of a primer and by *de novo* initiation were examined using polyC as template in the presence or absence of primer.

As shown in Figure 4, the rate of *de novo* RNA synthesis by the enzyme in the absence of primer was ~5-10 fold lower than the primer-dependent elongation reaction at GTP substrate concentrations of 0.5 to 2 μM. In this regard, a lag phase in the RNA synthesis rate was observed at low GTP concentration for the reaction containing no primer (Figure 4). Thus, the two reactions catalyzed by the same enzyme displayed two different substrate concentration-dependent patterns, as shown in Figure 4. These data suggest that addition of the first few nucleotides into the RNA product would be the rate limiting step during the viral genome replication, confirming that the RNA initiation reaction of the polymerase requires high substrate concentration, as seen with other viral RNA-dependent RNA polymerases.

Example 3
Demonstration of a Uridylate Residue As a Replication Initiation Site in the HCV Genome

We demonstrated that a uridylate residue located in the 3'-UTR of HCV positive strand RNA is a replication initiation site for primer-independent *de novo* RNA synthesis in the HCV genome as follows.

Preparation of an HCV RNA fragment for in vitro RNA replication--To prepare an RNA template containing the HCV 3'-UTR, an HCV genotype-1 a cDNA fragment (472 nt) was subcloned into a pSPT19 vector using standard recombinant DNA techniques (Sambrook, J. et al. *Molecular Cloning: A Laboratory Manual*, Second Ed., Cold Spring Harbor Lab. Press, CSH, NY), pp 1.21-1.105 (1989)). The resulting plasmid contained the HCV 3'-UTR and a partial coding sequence of NS5B (Figure 5). The transcription of this fragment was under control of the bacteriophage T7 promoter in a sense orientation.

The plasmid was then linearized by endonuclease *Bsm*I (Kolykhalov, A. A. et al. *Science* 277, 570-574 (1997)), and the DNA was extracted with phenol and chloroform and precipitated with ethanol. The purified DNA was used as the template for an *in vitro* transcription reaction to generate the expected RNA under the conditions provided by the manufacturer. To obtain labeled RNA fragments, [α-^{32}P]-CTP was added into the transcription reactions. After a 60 min incubation at 37^{o}C, the transcription reactions were mixed with RNA loading buffer and subjected to separation on a 1% agarose gel. After electrophoresis, the RNA products were detected by ethidium bromide staining and then eluted from the gel using Ultrafree-DA DNA extraction kit (Millipore). To prepare molecular markers, a 1 kb DNA ladder was labeled

by T4 polynucleotide kinase under the conditions provided by the manufacturer.

HCV polymerase assays--Full length NS5B polymerase from HCV genotype-1b was expressed in *E. coli* and purified to homogeneity by sequential chromatographic columns as described above. A typical primer-dependent HCV NS5B reaction was performed in a buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 10 μg/ml poly A template, 1 μg/ml $oligoU_{12}$ primer, and 10 μM UTP/[α-^{32}P]-UTP (specific activity: 4,500 cpm/pmol), and purified NS5B at 2 ng/μl. The other three homopolymeric RNAs were analyzed under the same conditions with corresponding oligonucleotide primers and appropriate substrates. Reactions were terminated at the time specified by the addition of 0.2 ml of 50 mM EDTA and then filtered through a nitrocellulose membrane using a 96 well minifold. The membrane was washed in 2X SSC buffer to remove the unused radiolabeled UTP substrate. Radioactive materials bound to the filter membrane were quantitated using a scintillation counter.

The primer-independent RNA synthesis reactions for the HCV NS5B polymerase were carried out under the conditions described above with various RNA templates and ribonucleotides at the concentrations as indicated, except that primer was absent. For reactions using homopolymeric RNAs as templates, the corresponding nucleotide substrate, ^{32}P-labeled at either α- or γ-position, was used at the concentrations indicated. Incorporation of the radiolabeled nucleotide was quantitated by scintillation counting as described above. For RNA synthesis reactions using the HCV RNA fragment as template, all four nucleotides were included, but with only one ^{32}P-radiolabeled at either the

α- or γ- position. The polymerase reactions were carried out in triplicate and terminated at the time specified. The RNA products were either quantitated by scintillation counting as described above, or treated and detected by sequencing gels as described below.

Analysis of RNA products by sequencing gels--To detect the RNA products by gel electrophoresis, the polymerase reaction mix was treated with proteinase K at 37°C for 30 min in a buffer containing 50 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5% SDS. The labeled RNA products were extracted by phenol-chloroform and precipitated with ethanol. The extracted RNA samples were mixed with the loading buffer, heated at 100°C for 5 min, and then loaded on to denaturing 6% polyacrylamide/8 M urea gels for separation. After electrophoresis at 50 watts for 50 min, the gels were fixed, dried, and exposed against X-ray film.

The results were as follows.

De novo RNA synthesis by purified HCV NS5B--As disclosed in Example 1, above, purified non-tagged HCV NS5B was able to catalyze RNA synthesis from homopolymeric RNA templates in the presence of the complementary primers. Of the four homopolymer RNAs tested, polyA was found to be the most efficient template for the enzyme to synthesize RNA through primer elongation (Figure 1A). As a comparison, reactions containing no primer were also performed under the same conditions (Figure 1B). Surprisingly, in the absence of primer, radiolabeled RNA products were detected in the reactions using polyC as the template (Figure 1B). A low level of radiolabeled RNA products was also detected in the reactions using polyU, indicating that NS5B specifically recognized a polypyrimidine template for primer-independent

RNA synthesis. As no RNA products were detected from the reactions containing polyA/UTP or polyG/CTP as template/substrate in the absence of primers (Figure 1B), we concluded that the resulting RNA products were not produced via any terminal transferase activity.

The resulting radiolabeled products were confirmed to be RNA by virtue of their sensitivity to RNase A treatment (data not shown). Under identical conditions, *de novo* RNA synthesis was not found in reactions containing the bacteriophage T7 and SP6 RNA polymerase (data not shown), indicating the reaction catalyzed by the HCV polymerase was specific. In addition, the purified genotype-1b NS5B with a C-terminal deletion of 52 amino acids and the NS5B polymerase from genotype-2a were also able to catalyze *de novo* RNA synthesis using polyC as the template (data not shown).

To exclude the possibility that the RNA synthesis catalyzed by HCV NS5B was directed by RNA or DNA primers that contaminated the enzyme preparation, we performed the reaction using [γ-^{32}P]-GTP as the labeled substrate. If HCV NS5B acted through a *de novo* mechanism, the first nucleotide incorporated into the RNA products would be a guanosine triphosphate (GTP) and thus the RNA products would be labeled. However, if the enzyme catalyzed primer-dependent RNA elongation, the resulting RNA products would not be labeled as only GMP would be incorporated. The data, as seen in Figure 3A, clearly showed that the RNA products were ^{32}P-labeled, indicating the presence of GTP in the products. No labeled products were detected when polyA was used as the template under identical conditions (Figure 3A), confirming that the *de novo* RNA synthesis was template-specific. In the absence of primer, RNA products were not detected from

the reactions containing polyA/UTP as template/substrate, excluding the possibility of terminal transferase activity associated with the HCV NS5B polymerase (Figures 1B and 3B). Taken together, these data strongly suggested that the purified HCV NS5B enzyme catalyzes RNA synthesis via a *de novo* mechanism.

Characterization of de novo RNA synthesis catalyzed by HCV NS5B--To understand further this primer-independent *de novo* RNA synthesis reaction, we carried out a series of experiments. We found that the NS5B polymerase catalyzed the *de novo* RNA synthesis reaction in an enzyme-concentration-dependent manner (Figure 2A). Substrate incorporation increased linearly in the first 60 min of the reaction and continued to increase up to 6 hrs (Figure 3B). Divalent cations, such as Mg^{++}, were required for this novel reaction, and the optimum temperature was determined to be 25-30 ^{o}C (data not shown), similar to the conditions reported for the primer-dependent RNA synthesis.

Next, we tested the effect of the template concentration on RNA synthesis efficiency. The rate of *de novo* RNA synthesis for HCV NS5B was dependent on the template concentration and reached the maximum when ~1 µg/ml template (polyC or polyU) was used under the conditions described (results not shown). However, the reaction rate decreased significantly when higher template concentrations were used; for example, almost no RNA synthesis was observed when the polyU concentration was >10 µg/ml (data not shown). As a comparison, polyA and polyG, within a wide range of concentrations tested, could not be utilized by the enzyme for *de novo* RNA synthesis (data not shown).

These RNA initiation reactions were also dependent on substrate concentration. When polyC was used as the template and GTP as the substrate, HCV NS5B catalyzed the reaction in the absence of primer with a Km and Vmax values of 70±15 μM and 246±18 pmol/hr/μg enzyme, respectively. With polyU as the template, NS5B had a Km value of 1300 μM with respect to ATP as the substrate, which was about 18-fold higher than that for GTP. However, the Vmax for ATP was about 153 pmol/hr/μg enzyme, only slightly lower than that for GTP as substrate. Therefore, the NS5B catalytic efficiency, expressed as Vmax/Km, was approximately 30-fold higher with polyC as template than with polyU.

De novo RNA synthesis catalyzed by HCV NS5B using an HCV RNA template--To determine if the HCV NS5B polymerase could catalyze *de novo* RNA synthesis from a HCV RNA template, we generated an RNA fragment from a HCV genotype-1a cDNA clone as described in the Materials and Methods section, above. This RNA fragment, as illustrated in Figure 5A, was 472 nucleotides in length and contained 47 nts from the vector, a partial NS5B coding sequence (154 nt), and the entire HCV 3'-UTR comprised of a short sequence of 40 nts followed by a poly(U)/polypyrimidine tract of 133 nts and an additional 98 nts (3'-X sequence) as shown in Figure 5A.

We then performed the HCV RNA polymerase assay using the HCV RNA fragment as the template and analyzed the RNA products on sequencing gels. As shown in Figure 5B, the major products of the reactions were ~370 nucleotides in length, i.e., about 100 bases smaller than the template. Meanwhile, several minor RNA products, smaller than the template and the major RNA product, were present in the reaction, indicating various stages in RNA synthesis. In addition, reactions performed without the template or enzyme

did not generate any RNA products (not shown). Again, we found that RNA synthesis efficiency was dependent on template concentration; higher template concentrations (>3 μg/ml) had a negative impact on the NS5B activity. Because the HCV NS5B polymerase has been reported to synthesize RNA by self-priming or copy-back of the HCV RNA template (Behrens, S. E. et al. *EMBO J.* 15, 12-22 (1996); Lohmann, V. et al. *J Virol.* 71, 8416-8428 (1997); Yamashita, T. et al. *J. Biol. Chem.* 273, 15479-15486 (1998)), we performed the following experiments to define the mechanism used by the viral polymerase. First, we used a ^{32}P-labeled HCV RNA template and performed the reaction using only cold nucleotides. If the RNA products resulted from self-priming or copy-back of the template, a change in the molecular size of the radiolabeled RNA template would be evident. As shown in Figure 5C, the polymerase reactions using unlabeled substrates did not change the size of the HCV RNA template. Thus, it is unlikely that the RNA products synthesized by HCV NS5B were mediated by a template self-priming mechanism. The data also ruled out the possibility that the observed RNA products were derived from degradation of any larger molecular RNA products including the template.

For further verification of this novel mechanism, γ-^{32}P labeled nucleotides were employed in the reactions for the reasons described above. Based on the fact that NS5B catalyzed *de novo* RNA initiation using only polyC and polyU as templates, we tested the incorporation of both [γ-^{32}P]-GTP and [γ-^{32}P]-ATP in the assays with HCV RNA as the template. As shown in Figure 6, no ^{32}P-labeled RNA products were obtained if [γ-^{32}P]-GTP was used in the assay. However, when [γ-^{32}P]-ATP was provided, newly synthesized RNA products were observed, with the major product shown as

the same size to that produced using [α-^{32}P]-ATP (Figures 5 and 6). On the basis of these data, we concluded that the purified HCV NS5B enzyme catalyzed *de novo* RNA synthesis from the HCV RNA template with a major product of approximately 360 bases. Since the viral enzyme selectively added ATP as the first nucleotide of the nascent RNA products (Figure 6), we conclude that HCV NS5B initiated the RNA replication by recognizing a uridylate present in the HCV RNA fragment.

The foregoing data demonstrate that purified HCV NS5B polymerase can catalyze *de novo* RNA synthesis in the absence of primers. The initial experiments were performed using homopolymeric RNAs as templates. Since homopolymer RNAs were unlikely to form intramolecular hairpins, the RNA products would not result from elongation of the template from a self-priming mechanism. Taken together with the results that RNA products were labeled in the reactions containing [γ-^{32}P]-GTP and polyC as the template, HCV NS5B appears to catalyze RNA synthesis not only through primer-dependent elongation as described previously, but also through a *de novo* initiation mechanism. This novel mechanism associated with the HCV NS5B polymerase includes a relevant HCV RNA template.

As the enzyme catalyzes *de novo* RNA synthesis using only polyC and polyU as templates, but not polyA and polyG, the enzyme appears to specifically recognize pyrimidine residues for initiation. Consistent with this observation, several other viral RNA polymerases have been reported to initiate RNA synthesis from a cytidylate residue (Kao, C. C. et al. *J. Virol.* 70, 6826-6830 (1996); Kao, C. C. et al. *Virology* 253, 1-7 (1999)). We did observe that the NS5B catalytic efficiency with polyC as template was ~30 higher

than that with polyU (Figure 1). However, when the HCV RNA fragment was used as the template, the first nucleotide added in the nascent RNA products was found to be an ATP, indicating that the HCV polymerase recognized a uridylate present in the HCV RNA for initiation. Thus, it is unclear to date how the viral enzyme selectively recognizes a uridylate, rather than a cytidylate, present in the template representing the 3'-UTR of the plus strand HCV RNA genome.

An interesting question raised by these data is which uridylate present in the HCV RNA was the initiation site. As mentioned, the HCV RNA template used in these studies contained the entire 3'-UTR of the plus strand HCV RNA. It is known that the HCV 3'-UTR contains three major elements: a short sequence of ~40 nts; a polyU/polypyrimidine tract; and a highly conserved heteropolymeric 3'-X sequence of 98 nucleotides. Recently, both the polyU/polypyrimidine tract and the conserved 3'-X sequence were reported to be essential for HCV infection in chimpanzees (Yanagi, M. et al. *Proc. Natl. Acad. Sci. USA* 96, 2291-2295 (1999)). The importance of the 3'-UTR polyU/polypyrimidine tract and 3'-X sequence during HCV infection perhaps is interpreted by the following hypotheses. Given the facts that the HCV polymerase utilized polyU to initiate RNA synthesis and that the major RNA product was about 370 nucleotides in length, which was ~100 nucleotides shorter than the template (Figure 5), it is possible that the enzyme recognized a uridylate present in the polyU/polypyrimidine tract. If true, however, it is not clear how replication of the 3'-X sequence would occur *in vivo*. Another possibility is that the final uridylate present in the 3'-X sequence, which is the last nucleotide of the plus strand HCV RNA genome, served as the initiation site. After initiation, the polymerase, without the presence of other viral and/or cellular proteins, might skip the secondary-structure-rich

3'-X sequence to continue RNA elongation. Further characterization of the NS5B *de novo* RNA synthesis from the HCV RNA genome is needed in order to determine which of these two mechanisms is occurring and the role, if any, of the other proposed replicase subunits.

The data presented herein demonstrate that purified HCV NS5B alone is able to initiate *de novo* RNA synthesis from the authentic HCV 3'-UTR, a mechanism expected to occur during HCV genome replication *in vivo*. This mechanism would be expected to be applicable to other viruses in the Flaviviridae family. Supporting this conclusion is the recent description of the viral bovine diarrhea virus RNA polymerase, which catalyzes *de novo* RNA synthesis from chemically synthesized RNAs and DNAs (Kao, C. C. et al. *Virology* 253, 1-7 (1999)). On the other hand, this mechanism is very different from that described for other plus-strand RNA viruses such as poliovirus in the picornavirus family, which have been shown to use protein-primed RNA initiation to start their genome replication (Paul, A.V. et al. *Nature* 393, 280-284 (1998)). From this perspective, it appears that the HCV NS5B polymerase is a dual functional enzyme, with both primase and polymerase activity, differing from the poliovirus RNA-dependent RNA polymerase.

Example 4

Different Metal Requirements for HCV NS5B RNA-Dependent Polymerase and Primase Activities

The data presented above demonstrate that HCV NS5B polymerase exhibits both RNA-dependent RNA polymerase and primase activities. With respect to polymerase activity,

purified NS5B has an absolute requirement for divalent cations, with Mg^{++} being the most preferred metal for this catalytic activity. With respect to the NS5B primase activity, similar studies have not been conducted. Interestingly, it has been reported recently that human DNA primase activity could be stimulated by Mn^{++} (Kirk, B.W. et al. *Biochemistry* 38:10126-10134 (1999)). Based on the results presented herein and those of others, it is possible that the polymerase and primase activities of the enzyme may have different metal requirements. To investigate this possibility, we examined the effects of Mn^{++} and Mg^{++} ions on the NS5B polymerase and primase activities.

Materials--The homopolymeric RNAs were purchased from Pharmacia. Oligonucleotide RNA primers were custom synthesized by CyberSyn (Lenni, PA). Radiolabeled nucleotides were purchased from Amersham and NEN. HCV NS5B protein was purified to homogeneity by sequential chromatographic columns as described above.

HCV NS5B assays--NS5B RNA-dependent RNA polymerase activity was measured using homopolymeric polyA and oligoU_{12} as template and primer, respectively. This primer-dependent NS5B reaction was performed in a buffer containing 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM DTT, 2 µg/ml polyA template, 1 µg/ml oligoU_{12} primer, 50 µM UTP/[α-32P]-UTP (specific activity: 2,000-3,000 cpm/pmol), purified NS5B at 2 µg/ml, and various concentrations of $MgCl_2$ and $MnSO_4$ as indicated. Reactions were terminated at the time specified by the addition of 0.2 ml of 50 mM EDTA, and then filtered through a nitrocellulose membrane using a 96-well minifold dot blotter. The membrane was washed in 2X SSC buffer to remove the unused radiolabeled UTP substrate. Radioactive

materials bound to the filter membrane were quantitated using a scintillation counter.

HCV NS5B primase activity was measured using homopolymeric polyC or polyU as the template in the absence of any primer. These primer-independent RNA synthesis reactions using HCV NS5B were carried out under the same conditions described above, except that polyC or polyU was used as template, and GTP or ATP as substrate, respectively, and no primer was present.

Results--The RNA-dependent polymerase and primase activities associated with HCV NS5B were each dependent on the concentration of the divalent cation Mn^{++} or Mg^{++} (Figure 7). In the case of the NS5B primer-dependent RNA synthesis reaction, both Mn^{++} and Mg^{++}, when used at different optimum concentrations, produced very similar maximal velocities.

In the case of the primer-independent primase reaction, Mn^{++} was preferred over Mg^{++} ion, with an approximately 19-fold greater activity when Mn^{++} was used in the reaction (Figure 7). The maximal primase activity seen with Mn^{++} was 3-fold higher than that with Mg^{++} as the divalent cation (Figure 7). In addition, *de novo* RNA synthesis from polyU template was also more efficient using Mn^{++} as compared to Mg^{++} over the same concentration range as that shown in Figure 7 (data not shown).

Taken together, these data suggest that HCV NS5B primase and polymerase activities have different metal requirements. Similar to human DNA primase, HCV NS5B RNA primase activity was stimulated by Mn^{++} ion to a greater extent than by Mg^{++} ion.

The greater effectiveness of Mn^{++} as a cofactor in the primase reaction can be employed in the assays and methods described herein, including those designed to discover compounds that inhibit primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerases such as the HCV polymerase NS5B and other similar RNA polymerases. Under conditions like those employed in this example, the optimal concentration of Mn^{++} salt is in the range of from about 0.5 mM to about 20 mM, preferably from about 2.5 mM to about 15 mM, more preferably from about 5 mM to about 10 mM. Any Mn^{++} salt can be used regardless of the nature of the anion. It should be noted that Mn^{++} salts can be used alone in reaction mixtures, or in combination with Mg^{++} salts.

In the absence of an efficient cell culture system for HCV replication, the *de novo* RNA initiation assays described herein facilitate investigation of the NS5B polymerase initiation requirements, as well as identification of the HCV RNA replication initiation sites *in vivo*. As HCV infection becomes an increasingly serious threat to human health, development of safe and specific antivirals against HCV is highly desired. The *de novo* RNA synthesis assays disclosed herein can be used to search for HCV polymerase initiation inhibitors. Unlike the assays described previously, the primer-independent assays could permit identification of inhibitors that specifically block the first step of RNA synthesis catalyzed by HCV polymerase.

The invention being thus described, it is obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications as would be obvious to one skilled

in the art are intended to be included within the scope of the following claims.

What Is Claimed Is:

1. A method of identifying a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase, comprising:

d) contacting an RNA template comprising an RNA synthesis initiation pyrimidine nucleotide with a flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the absence of said compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

e) contacting an RNA template as in a) with said flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the presence of said compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

f) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase.

2. The method of claim 1, wherein said RNA template is a synthetic RNA sequence.

3. The method of claim 2, wherein said synthetic RNA sequence comprises a sequence selected from the group consisting of a homopolyC template; a homopolyU template; a synthetic heteropolymeric RNA template comprising a cluster

of cytidylate nucleotides, a cluster of uridylate nucleotides, or a mixed cluster of cytidylate and uridylate nucleotides; and a synthetic or naturally occurring RNA template comprising an RNA synthesis initiation pyrimidine nucleotide.

4. The method of claim 3, wherein said RNA synthesis initiation pyrimidine nucleotide is a cytidylate nucleotide or a uridylate nucleotide.

5. The method of claim 1, wherein said RNA template comprises hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication.

6. The method of claim 5, wherein said fragment comprises the 3' untranslated region of positive or negative RNA strand hepatitis C virus genomic RNA.

7. The method of claim 5 or 6, wherein said RNA synthesis initiation pyrimidine nucleotide is a uridylate nucleotide.

8. The method of any one of claims 1-7, wherein said flavivirus RNA-dependent RNA polymerase is a hepatitis virus RNA-dependent RNA polymerase.

9. The method of claim 8, wherein said hepatitis virus is a hepatitis C virus.

10. The method of claim 9, wherein said hepatitis C virus is selected from the group consisting of hepatitis C virus genotype 1a, hepatitis C virus genotype 1b, hepatitis C virus genotype 2a, hepatitis C virus genotype 2b, progeny of any of the foregoing, and a mutant of any of the foregoing.

11. The method of claim 10, wherein said hepatitis C virus is a hepatitis C virus genotype 1b virus, or any progeny or mutant thereof.

12. The method of claim 11, wherein said hepatitis C virus RNA-dependent RNA polymerase is hepatitis C virus NS5B polymerase.

13. The method of claim 12, wherein said hepatitis C virus NS5B polymerase is produced recombinantly.

14. A method of identifying a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase, comprising:

d) contacting an RNA template comprising hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, with hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the absence of said compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

e) contacting said RNA template as in a) with said hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the presence of said compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

f) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a

compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase.

15. The method of claim 14, wherein said fragment comprises the 3' untranslated region of positive or negative strand hepatitis C virus genomic RNA.

16. A method of inhibiting primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase, comprising contacting said flavivirus RNA-dependent RNA polymerase with a polymerase-inhibiting effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by said polymerase,

wherein said compound is identified by a method comprising:

d) contacting an RNA template comprising an RNA synthesis initiation pyrimidine nucleotide with a flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the absence said compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

e) contacting an RNA template as in a) with said flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the presence of said compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

f) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by a flavivirus RNA-dependent RNA polymerase.

17. The method of claim 16, wherein said RNA template is a synthetic RNA sequence.

18. The method of claim 17, wherein said synthetic RNA sequence comprises a sequence selected from the group consisting of a homopolyC template; a homopolyU template; a synthetic heteropolymeric RNA template comprising a cluster of cytidylate nucleotides, a cluster of uridylate nucleotides, or a mixed cluster of cytidylate and uridylate nucleotides; and a synthetic or naturally occurring RNA template comprising an RNA synthesis initiation pyrimidine nucleotide.

19. The method of claim 18, wherein said RNA synthesis initiation pyrimidine nucleotide is a cytidylate nucleotide or a uridylate nucleotide.

20. The method of claim 16, wherein said RNA template comprises hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication.

21. The method of claim 20, wherein said fragment comprises the 3' untranslated region of positive or negative RNA strand hepatitis C virus genomic RNA.

22. The method of claim 20 or 21, wherein said RNA synthesis initiation pyrimidine nucleotide is a uridylate nucleotide.

23. The method of any one of claims 16-22, wherein said flavivirus RNA-dependent RNA polymerase is a hepatitis virus RNA-dependent RNA polymerase.

24. The method of claim 23, wherein said hepatitis virus is a hepatitis C virus.

25. The method of claim 24, wherein said hepatitis C virus is selected from the group consisting of hepatitis C virus genotype 1a, hepatitis C virus genotype 1b, hepatitis C virus genotype 2a, hepatitis C virus genotype 2b, progeny of any of the foregoing, and a mutant of any of the foregoing.

26. The method of claim 25, wherein said hepatitis C virus is a hepatitis C virus genotype 1b virus, or any progeny or mutant thereof.

27. The method of claim 26, wherein said hepatitis C virus RNA-dependent RNA polymerase is hepatitis C virus NS5B polymerase.

28. The method of claim 27, wherein said hepatitis C virus NS5B polymerase is produced recombinantly.

29. A method of inhibiting primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase, comprising contacting said hepatitis C virus RNA-dependent RNA polymerase with a polymerase-inhibiting effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by said polymerase,

wherein said compound is identified by a method comprising:

d) contacting an RNA template comprising hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, with hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the absence of said compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

e) contacting said RNA template as in a) with said hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the presence of said compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

f) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase.

30. The method of claim 29, wherein said fragment comprises the 3' untranslated region of positive or negative RNA strand hepatitis C virus genomic RNA.

31. A method of inhibiting replication of a flavivirus, comprising contacting said flavivirus with an antiviral effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerase,

wherein said compound is identified by a method comprising:

d) contacting an RNA template comprising an RNA synthesis initiation pyrimidine nucleotide with a flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the absence said compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

e) contacting an RNA template as in a) with said flavivirus RNA-dependent RNA polymerase in the absence of a primer and in the presence of said compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

f) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by flavivirus RNA-dependent RNA polymerase.

32. The method of claim 31, wherein said RNA template is a synthetic RNA sequence.

33. The method of claim 32, wherein said synthetic RNA sequence comprises a sequence selected from the group consisting of a homopolyC template; a homopolyU template; a synthetic heteropolymeric RNA template comprising a cluster of cytidylate nucleotides, a cluster of uridylate nucleotides, or a mixed cluster of cytidylate and uridylate nucleotides; and a synthetic or naturally occurring RNA

template comprising an RNA synthesis initiation pyrimidine nucleotide.

34. The method of claim 33, wherein said RNA synthesis initiation pyrimidine nucleotide is a cytidylate nucleotide or a uridylate nucleotide.

35. The method of claim 31, wherein said RNA template comprises hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication.

36. The method of claim 35, wherein said fragment comprises the 3' untranslated region of positive or negative RNA strand hepatitis C virus genomic RNA.

37. The method of claim 35 or 36, wherein said RNA synthesis initiation pyrimidine nucleotide is a uridylate nucleotide.

38. The method of any one of claims 31-37, wherein said flavivirus RNA-dependent RNA polymerase is a hepatitis virus RNA-dependent RNA polymerase.

39. The method of claim 38, wherein said hepatitis virus is a hepatitis C virus.

40. The method of claim 39, wherein said hepatitis C virus is selected from the group consisting of hepatitis C virus genotype 1a, hepatitis C virus genotype 1b, hepatitis C virus genotype 2a, hepatitis C virus genotype 2b, progeny of any of the foregoing, and a mutant of any of the foregoing

41. The method of claim 40, wherein said hepatitis C virus is a hepatitis C virus genotype 1b virus, or any progeny or mutant thereof.

42. The method of claim 41, wherein said hepatitis C virus RNA-dependent RNA polymerase is hepatitis C virus NS5B polymerase.

43. The method of claim 42, wherein said hepatitis C virus NS5B polymerase is produced recombinantly.

44. A method of inhibiting the replication of hepatitis C virus, comprising contacting said hepatitis C virus with an antiviral effective amount of a compound that inhibits primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase,

wherein said compound is identified by a method comprising:

d) contacting an RNA template comprising hepatitis C virus positive or negative strand genomic RNA, or a fragment thereof required for hepatitis C virus RNA genome replication, with hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the absence of said compound under conditions permitting RNA synthesis, and determining the amount of RNA product thus formed;

e) contacting said RNA template as in a) with said hepatitis C virus NS5B RNA-dependent RNA polymerase in the absence of a primer and in the presence of said compound under the same conditions as in a), and determining the amount of RNA product thus formed; and

f) comparing the amount of RNA product formed in b) with that in a),

wherein any reduction in the amount of RNA product formed in b) compared with that formed in a) indicates a compound that is an inhibitor of primer-independent *de novo* RNA synthesis catalyzed by hepatitis C virus RNA-dependent RNA polymerase.

45. The method of claim 44, wherein said fragment comprises the 3′ untranslated region of positive or negative RNA strand hepatitis C virus genomic RNA.

FIG. 1A

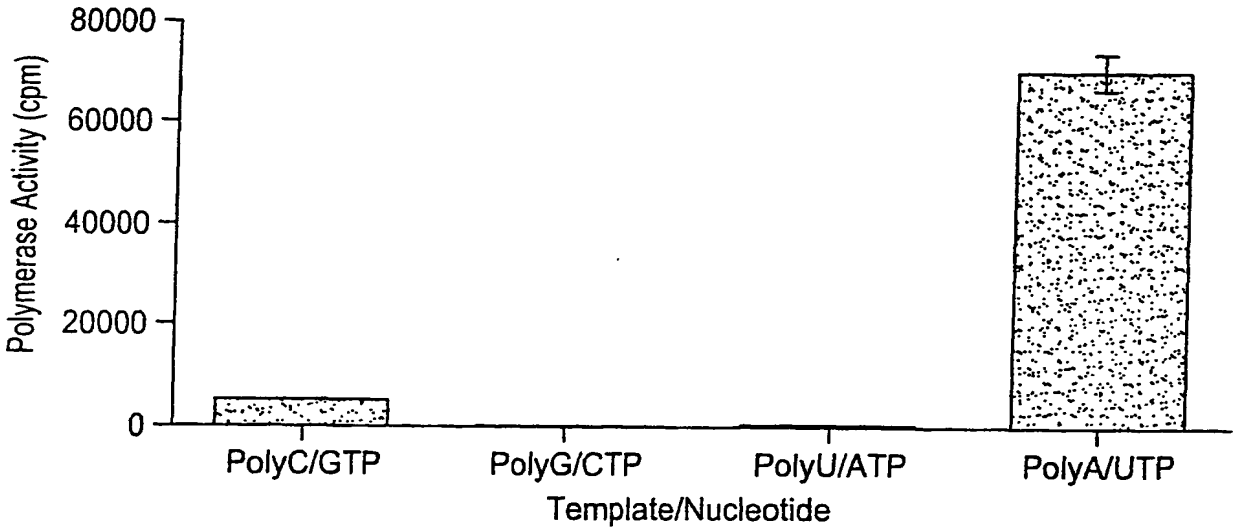


FIG. 1B

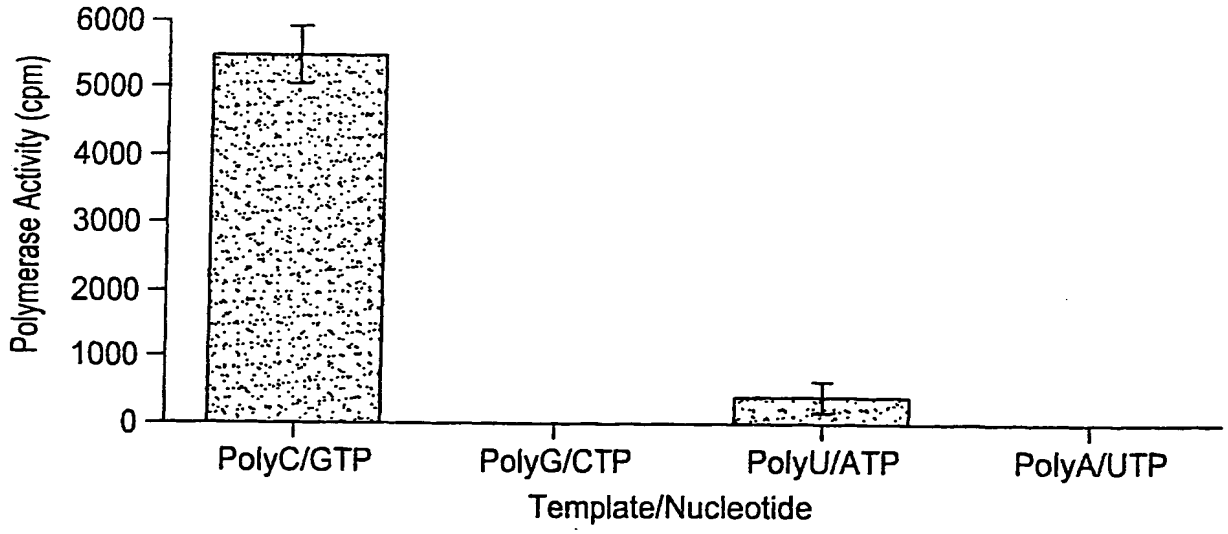


FIG. 2A

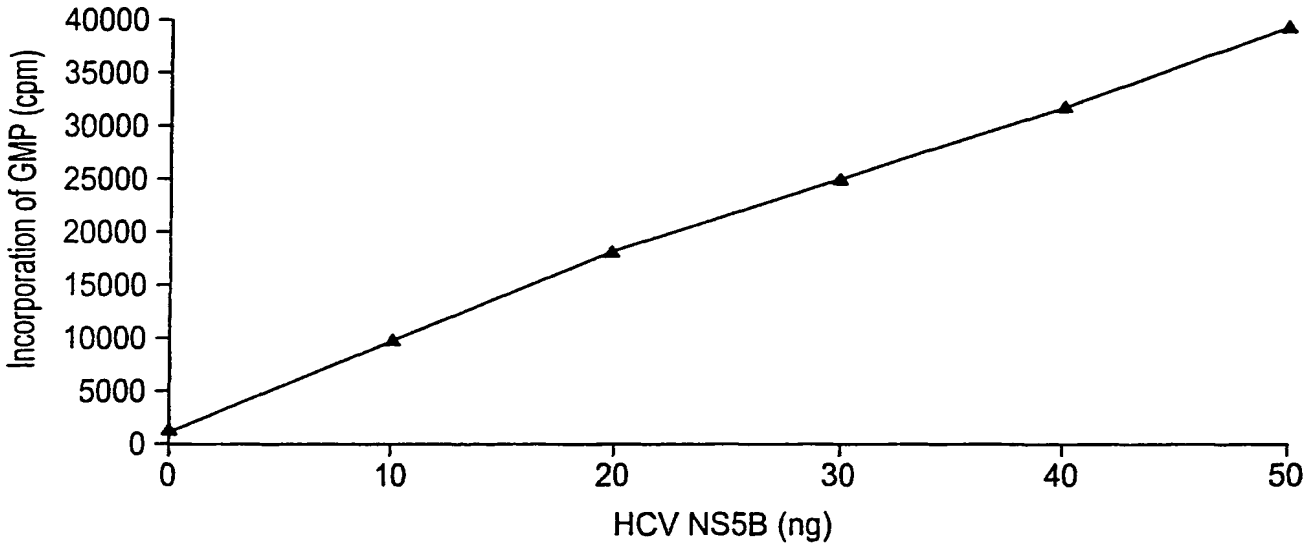


FIG. 2B

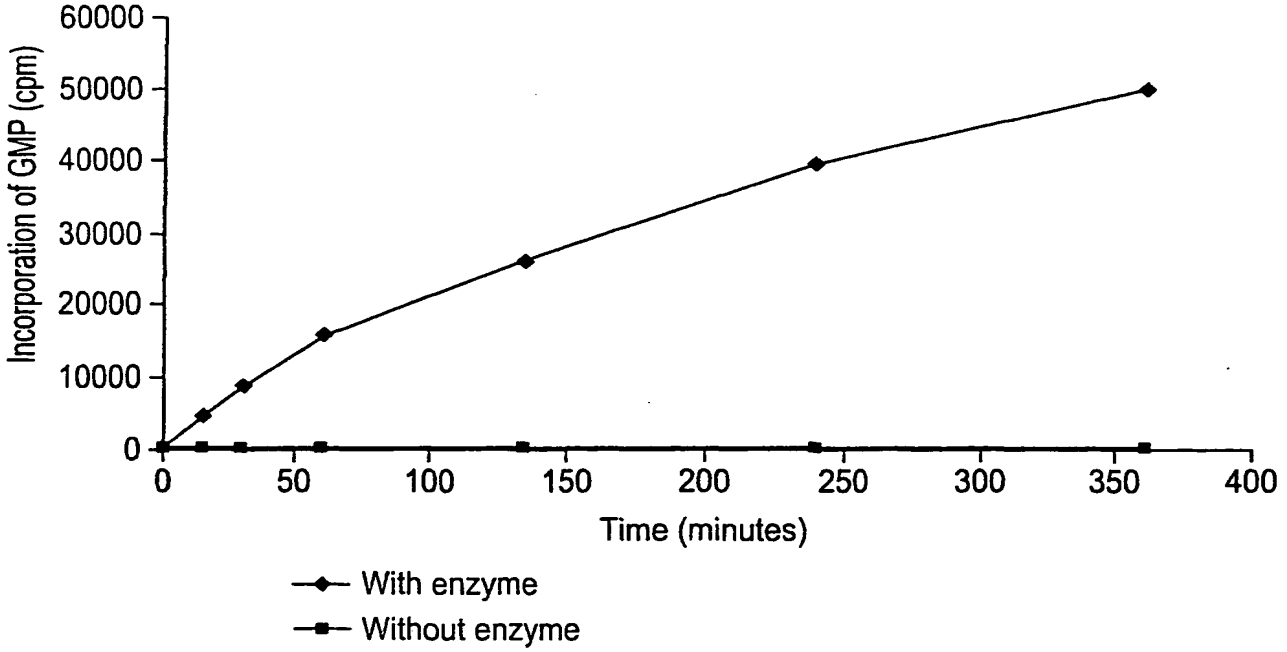


FIG. 3A

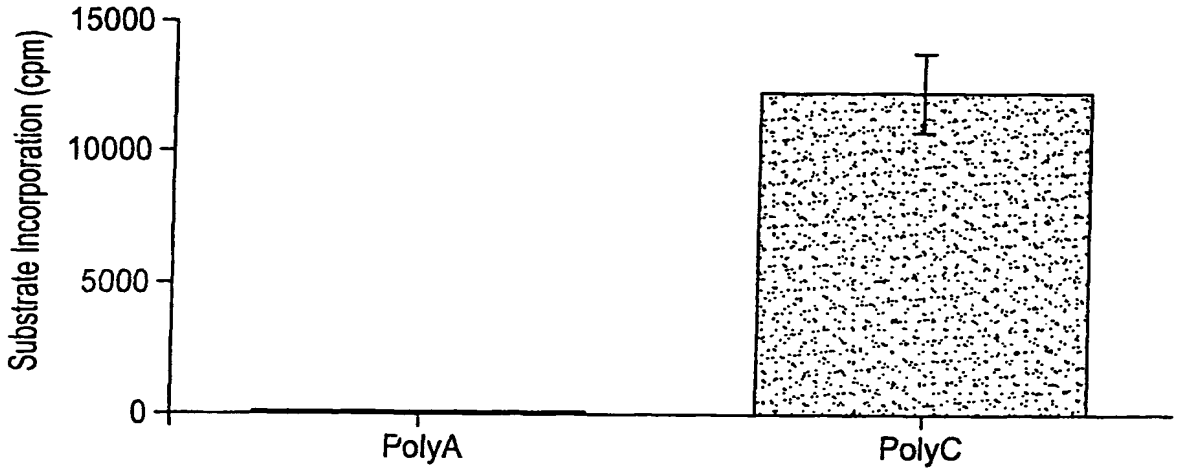


FIG. 3B

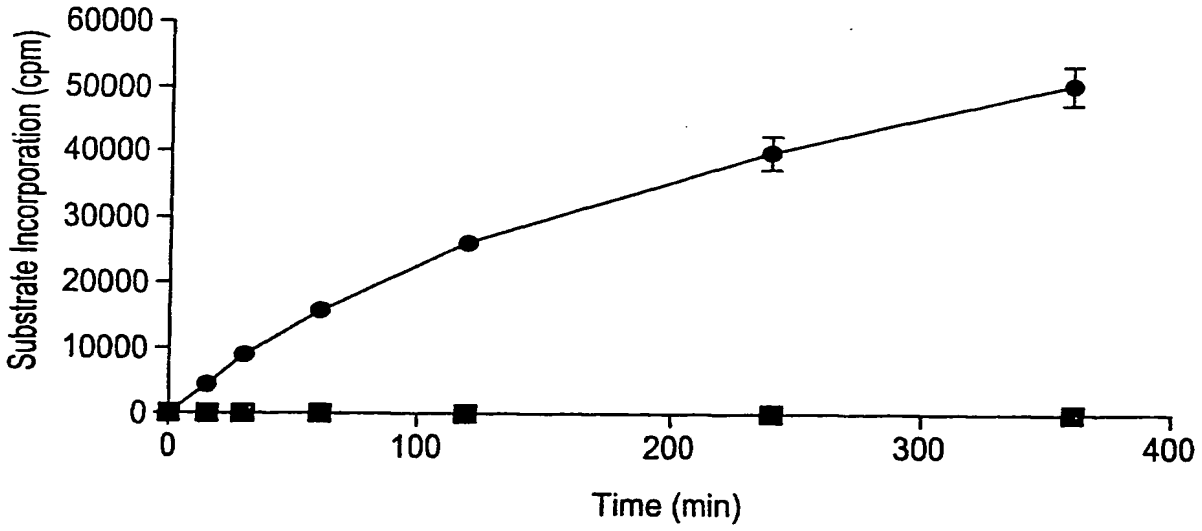


FIG. 4

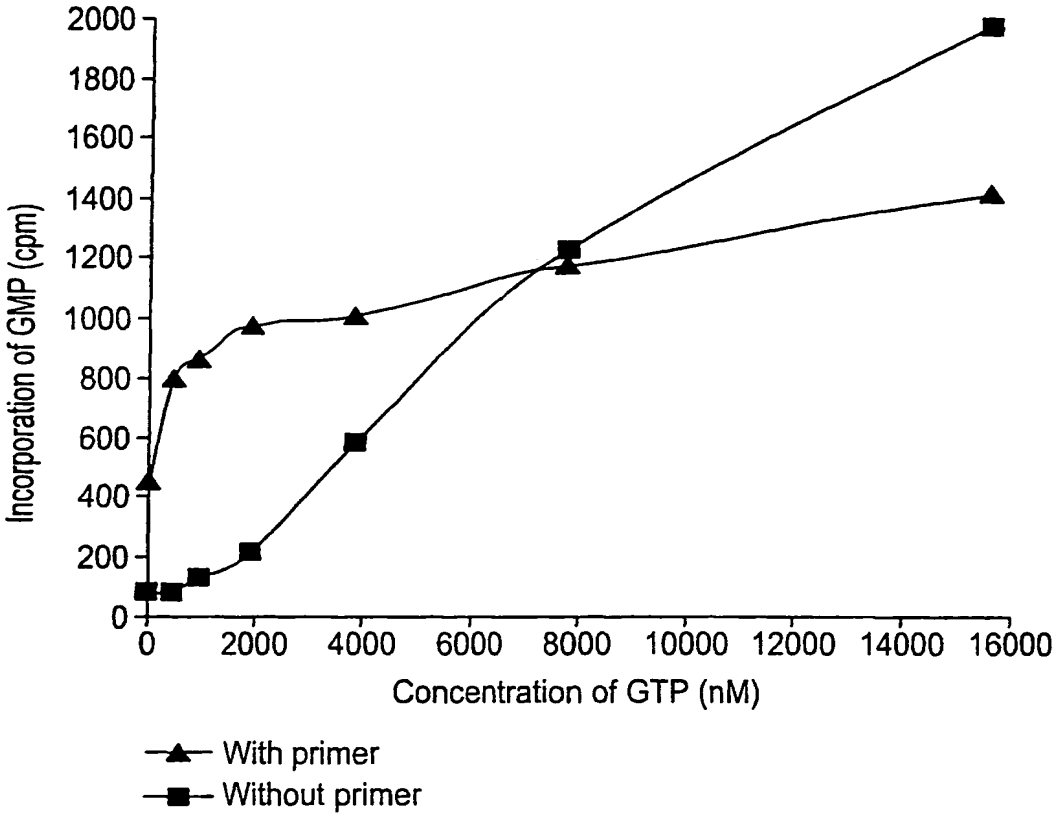
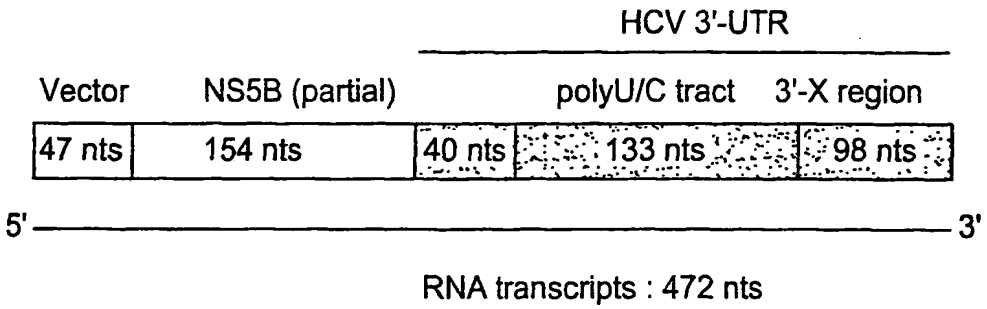


FIG. 5



RNA transcripts : 472 nts

FIG. 6B

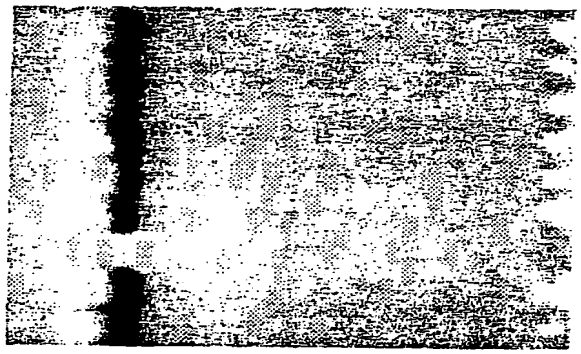


FIG. 6A

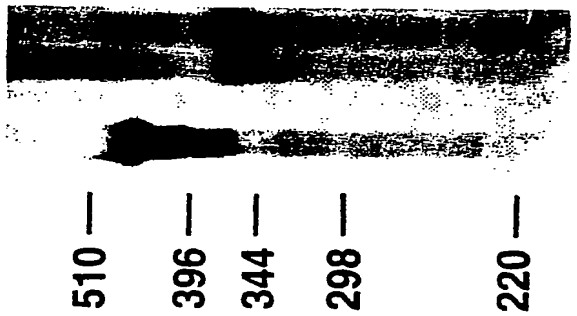


FIG. 7

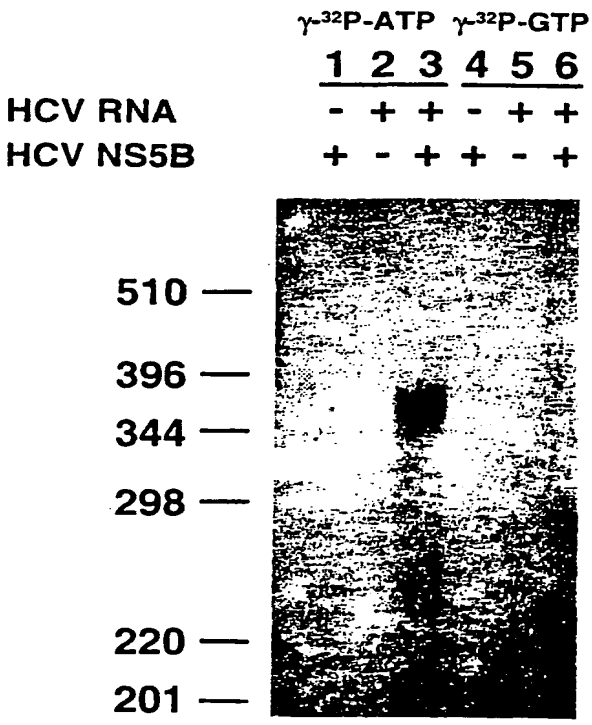
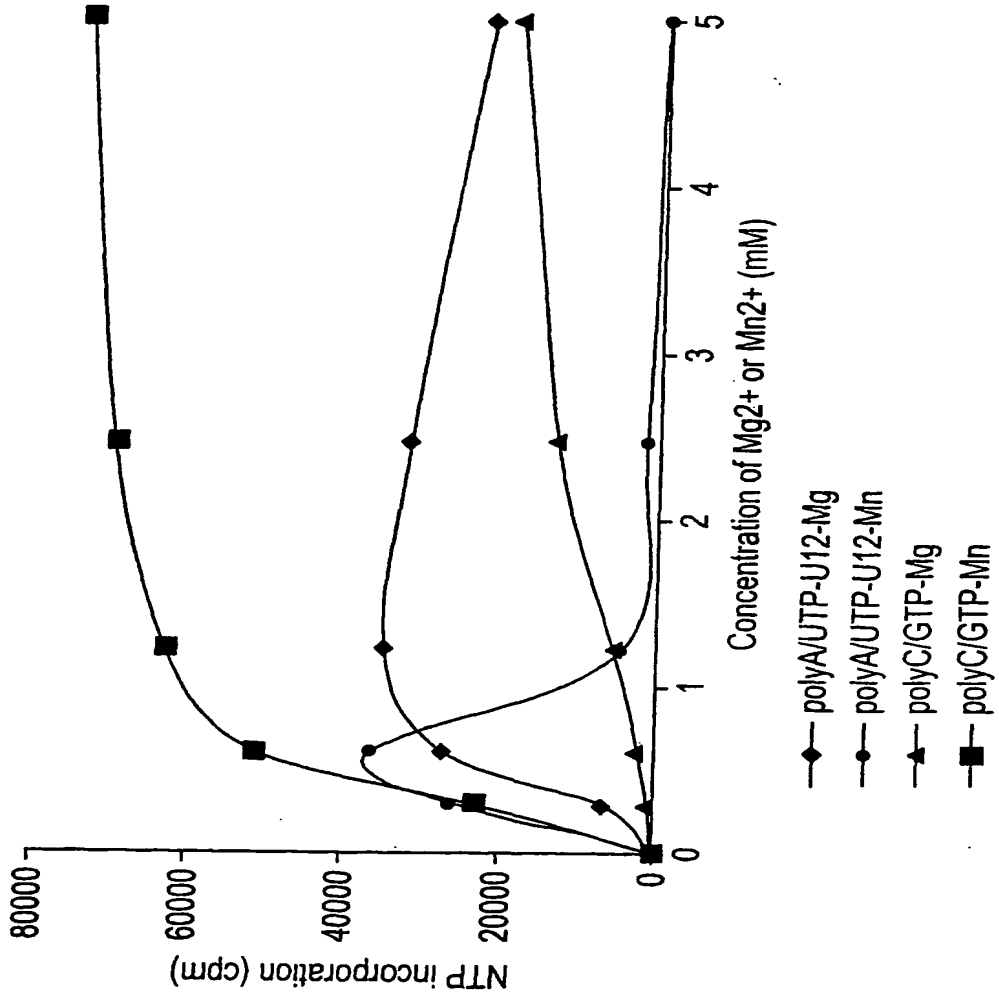


FIG. 8



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(54) Title: PRIMER-INDEPENDENT RNA SYNTHESIS CATALYZED BY HCV POLYMERASE

(57) Abstract: Provided are methods for identifying inhibitors of primer-independent hepatitis C virus RNA-dependent RNA polymerase *de novo* RNA synthesis activity, methods for inhibiting the polymerase using such inhibitors, and methods for inhibiting hepatitis C virus replication. These methods employ homopolyC templates; homopolyU templates; synthetic heteropolymeric RNA templates comprising a cluster of cytidylate nucleotides, a cluster of uridylate nucleotides, or a mixed cluster of cytidylate and uridylate nucleotides; and synthetic or naturally occurring RNA templates comprising an RNA synthesis initiation pyrimidine nucleotide. Reactions can be carried out in buffers containing Mn^{++} alone, or in combination with Mg^{++} ions.

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 99/29649

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/70 C12Q1/48 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 00 40759 A (KAO C CHENG ;ADVANCED RES & TECH INST (US)) 13 July 2000 (2000-07-13) see whole document, esp claims 2-9 --- -/--	1,2,4-6, 8,9, 12-17, 19-21, 23,24, 27-32, 34-36, 38,39, 42-45

[X] Further documents are listed in the continuation of box C.

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

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Interr nal Application No
PCT/US 99/29649

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	KAO C C ET AL: "De novo initiation of RNA synthesis by a recombinant flaviridae RNA-dependent RNA polymerase" VIROLOGY,ACADEMIC PRESS,ORLANDO,US, vol. 253, 1999, pages 1-7, XP002110174 ISSN: 0042-6822 the whole document	1,2,4-6, 8,9, 12-17, 19-21, 23,24, 27-32, 34-36, 38,39, 42-45
X	WO 96 37619 A (BEHRENS SVEN E ;ANGELETTI P IST RICHERCHE BIO (IT); FRANCESCO RAFF) 28 November 1996 (1996-11-28) page 10, line 33 -page 11, line 6; claim 7	1-45
X	YUAN Z -H ET AL: "EXPRESSION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF HCV RNA POLYMERASE" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,US,ACADEMIC PRESS INC. ORLANDO, FL, vol. 232, no. 1, 1997, pages 231-235, XP000877460 ISSN: 0006-291X the whole document	1-45
A	LOHMAN V ET AL: "Biochemical and kinetic analyses of NS5B RNA-dependent RNA polymerase of the hepatitis C virus" VIROLOGY, vol. 249, 1998, pages 108-18, XP002141668 the whole document	1-45
A	LOHMANN V ET AL: "BIOCHEMICAL PROPERTIES OF HEPATITIS C VIRUS NS5B RNA-DEPENDENT RNA POLYMERASE AND IDENTIFICATION OF AMINO ACID SEQUENCE MOTIFS ESSENTIAL FOR ENZYMATIC ACTIVITY" JOURNAL OF VIROLOGY,THE AMERICAN SOCIETY FOR MICROBIOLOGY,US, vol. 71, no. 11, November 1997 (1997-11), pages 8416-8428, XP000877461 ISSN: 0022-538X the whole document	1-45
A	WO 97 12033 A (UNIV EMORY) 3 April 1997 (1997-04-03) whole document, especially page 14, paragraph 2; and claims 24, 25.	1-45

2

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INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Inter nal Application No
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